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Abstract

Since the 2000s, a number of agents collectively known as novel oral anticoagulants (NOACs), directly acting oral anticoagulants (DOACs), or non-vitamin K antagonist oral anticoagulants have been introduced. NOACs are first-line drugs used for the treatment of venous thromboembolism and prevention of stroke in patients with atrial fibrillation. These drugs do not require the regular biochemical monitoring that is mandatory for traditional vitamin K antagonist (VKA) drugs, and they exhibit shorter half-lives and have a faster onset of action.

In current research work, three important NOACs, namely dabigatran (direct thrombin inhibitor), apixaban, rivaroxaban (direct factor Xa inhibitors), the non-vitamin K antagonist oral anticoagulants which are relatively newer in the market, have been selected for developing a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method in order to reliably estimate them in human plasma while adopting the latest regulatory norms and their applications to *in-vivo* bioequivalence studies.

Method for the estimation of free (non-conjugated) dabigatran and total dabigatran (non-conjugated plus conjugated dabigatran after complete alkaline cleavage of dabigatran glucuronides) from human plasma containing sodium citrate as anticoagulant using dabigatran D_4 as an internal standard (IS) was developed and validated with reproducible results. The extraction of analyte and IS was accomplished by solid phase extraction (SPE) technique. Chromatographic separations were achieved using Peerless basic C8, 150 mm x 4.6 mm, 5µm, HPLC column eluted at flow rate of 1 mL/min with mobile phase acetonitrile: methanol: 5 mM ammonium formate buffer (30:20:50, v/v/v). The run time of method was about 2.5 min with elution times of dabigatran and dabigatran D_4 at around 1.2 min. The multiple reaction monitoring transitions (Q_1/Q_3) were set at 472/289, 172 (m/z) for dabigatran and 476/293 (m/z) for dabigatran D_4. The calibration curves were linear ($r^2 \geq 0.99$) over the range of 1.04 - 406.49 ng/mL. The presented method was employed in bioequivalence study in healthy volunteers. Re-analysis of incurred sample resulted with >98% compliance indicating good assay precision of target analytes.

Method for the estimation of rivaroxaban from human plasma containing K_2EDTA as anticoagulant using rivaroxaban D_4 as an IS was developed and validated with reproducible results. The extraction of analyte and IS was accomplished by SPE technique. Chromatographic separations were achieved using Gemini C18, 150 mm x 4.6 mm, 5µm, HPLC column eluted at flow rate of 1.5 mL/min with mobile phase

acetonitrile: 5 mM ammonium acetate buffer (80:20 v/v). The run time of method was about 1.8 min with elution times at ~1.8 min. The multiple reaction monitoring transitions (Q_1/Q_3) were set at 436/145 (m/z) for rivaroxaban and 440/145 (m/z) for rivaroxaban D_4. The calibration curves were linear ($r^2 \geq 0.99$) over the range of 5.96 - 800.73 ng/mL. The presented method was employed in bioequivalence study in healthy volunteers. Re-analysis of incurred sample resulted with >98% compliance indicating good assay precision of target analytes.

Method for the estimation of apixaban from human plasma containing K_2EDTA as anticoagulant using apixaban $^{13}CD_3$ as an internal standard was developed and validated with reproducible results. The extraction of analyte and IS was accomplished by SPE technique. Chromatographic separations were achieved using Gemini C18, 50 mm x 4.6 mm, 3µm, HPLC column eluted at flow rate of 1.0 mL/min with mobile phase acetonitrile: 2 mM ammonium formate buffer (50:50 v/v). The run time of method was about 1.8 min with elution times at ~0.9 min. The multiple reaction monitoring transitions (Q_1/Q_3) were set at 460/443 (m/z) for apixaban and 464/447 (m/z) for apixaban $^{13}CD_3$. The calibration curves were linear ($r^2 \geq 0.99$) over the range of 1.01 - 280.16 ng/mL. The presented method was employed in bioequivalence study in healthy volunteers. Re-analysis of incurred sample resulted with >99% compliance indicating good assay precision of target analytes.

Keywords: Anticoagulant; NOACs; dabigatran; β-glucuronide; rivaroxaban, apixaban; LC–MS/MS; human plasma; pharmacokinetics; bioavailability; bioequivalence.

Chapter 1 Introduction

Anticoagulants are medicines that aid in the prevention of blood clots. They are given to people who are at high risk of getting clots in order to reduce their chances of developing serious conditions like strokes and heart attacks.

Since the 2000s, a number of agents collectively known as novel oral anticoagulants (NOACs), directly acting oral anticoagulants (DOACs), or non-vitamin K antagonist oral anticoagulants have been introduced and resolved these issues to a remarkable extent. It is at least as effective as traditional anticoagulants and is convenient to administer because it is given in fixed doses and does not require regular coagulation monitoring[1]. It has a consistent and predictable PK-PD profile[2]. NOACs have gained popularity in the market as a result of their advantages over traditional anticoagulants.

NOACs include four drugs, the first of which, dabigatran, a direct thrombin inhibitor, was approved by the FDA in 2010. Rivaroxaban, apixaban, and edoxaban are direct factor Xa inhibitors approved in 2011, 2012 and 2015, respectively[3]. In our current research, three NOACs (dabigatran rivaroxaban and apixaban) are included.

Bioavailability (BA)/ bioequivalence (BE) studies are conducted to assess the *in-vivo* evaluation of generic formulations and to obtain essential details in the overall data set to ensure that patients and physicians have access to safe and reliable drugs.

In a general prospective of BA/BE studies, bioanalysis is the subsequent step following clinical operations of the study and is executed with strict adherence to good laboratory practices (GLP), standard operating procedures, and specific regulatory requirements. Bioanalysis is a term generally used to describe the quantitative measurement of the active moiety and/or its metabolic product(s) in biological matrix. To produce reliable results that can be satisfactorily interpreted, bioanalytical methods used, should be well characterised, and fully validated and documented.

Chapter 2 Literature review

2.1 Drug profile of selected NOACs

The physico-chemical & pharmacological properties of selected NOACs are summarized in below table.

Characteristics	Dabigatran	Rivaroxaban	Apixaban
Chemical formula	$C_{25}H_{25}N_7O_3$	$C_{19}H_{18}ClN_3O_5S$	$C_{25}H_{25}N_5O_4$
Molecular weight	471.5	435.9	459.5
Solubility	Freely soluble in methanol, slightly soluble in ethanol, and sparingly soluble in isopropanol	Slightly soluble in organic solvents	Soluble in methanol
pKa[a]	4.0	13.6	13.12
pKa[b]	6.7	-1.6	-1.6
Protein binding (%)	~35	92-95	92-94
Volume of distribution (L)	50-70	~50	~21
Mechanism of Action	Direct thrombin inhibitors	Direct Xa Inhibitor	Direct Xa Inhibitor
Prodrug	Yes	No	No
Strengths (mg)	75, 110, and 150	2.5, 10, 15, and 20	2.5 and 5
Absolute Bioavailability (%)	3-7	80-100	~50
T_{max} (h)	1-2	2-4	3-4
Half Life ($T_{1/2}$ (h))	12-17	5-9	~12
Renal Clearance (%)	80	35	27
Drug Interactions	P-gp	Strong inhibitors of both CYP3A4 and P-gp	Strong inhibitors of both CYP3A4 and P-gp

[a] Strongest acidic; [b] strongest basic

2.2 Existing techniques for the quantitative determination of NOACs in biological matrix

Numerous methods of quantification are available to determine dabigatran[4-8], rivaroxaban[9-11] and apixaban[12-14] individually using LC-MS/MS methods. Most of the analytical methods alluded to were concentrated in the gradient mobile phase, requiring a long period of time and a high limit of quantification for individual quantification. For high-throughput analysis, a relatively long chromatographic run time may not be sufficient. In Bioanalysis, the preparation of sample in biological

matrix plays a significant role to evaluate the level of recovery of the analyte from the sample. For sample cleanup, the majority of these methods utilized protein precipitation (PP) technique[4-10,12,14]. The advantage of PP is that the method is simple, rapid and inexpensive and tendency to extract both hydrophilic and lipophilic compounds; however such precipitation leaves phospholipids on the analytical column, which can pollute the mass spectrometer's source, resulting in matrix effects and lower sensitivity. The interference can be predominantly critical when the volume of the biological sample is more than 50 µL and the procedure may give inaccurate results as far as a large number of samples to analyze. With this condition, criteria for the selection of extraction method were clear to adopt the SPE method for analysis. Two methods were found where SPE was used[11,13] but those failed to set the other parameters simpler for analysis.

Chapter 3 Rationale, aim and objective

3.1 Rationale

In current research work, three important NOACs, commonly known as novel oral anticoagulants namely dabigatran (direct thrombin inhibitor), apixaban, rivaroxaban (direct factor Xa inhibitors), the non-vitamin K antagonist oral anticoagulants which are relatively newer in the market, have been selected for developing a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method in order to reliably estimate them in human plasma while adopting the latest regulatory norms.

The science of bioequivalence evaluations for generics has been in place in most countries for more than 25 years with an established track record of therapeutic equivalence. These evaluation methods have been so successful in establishing generic drug standards that they are largely consistent across all major drug regulators worldwide. Consumers and healthcare professionals can reassured that generic drugs approved under these regulatory frameworks are bioequivalent and thus interchangeable with brand-name products.

The use of generic preparation of a therapeutically well-established active drug has to be justified by a well-designed, conducted and analyzed bioequivalence study. In today's drug research environment, extremely sensitive and selective approaches are necessary to quantify drugs in matrices such as blood, plasma, serum, or urine.

Based on a thorough literature review, it was decided to undertake the proposed research work, due to the inexistence of detailed research articles (in the public domain) dealing with the estimation of dabigatran, rivaroxaban, and apixaban in human plasma, using a highly specific and sensitive bioanalytical method, in line with the recent regulatory standards.

In addition to the inexistence of appropriate literature, a challenging task involved in development of a selective bioanalytical method with very high sensitivity, further motivated us to take up this work, which would expected to give benefit largely to the research community involved in development of the low cost and quality generic versions (as an alternative to the innovator's brand product) of the dabigatran, rivaroxaban, and apixaban for the benefit of the patient.

Hyphenated analytical techniques are mostly preferred by the pharma industry for development of the fast and cost effective methods since 1990s. LC-MS/MS has evolved to significant progress in quantitative analysis of drugs in biological fluids

because of its inherent characters like high specificity, speed, and sensitivity. It combines the separation capability of LC with the high sensitivity and selectivity of mass spectrometry, therefore, considered as method of choice in various fields like therapeutic drug monitoring, drug discovery/development and toxicology[15]. Thus, unequivocally LC-MS/MS was chosen as analytical technique in proposed research work.

Human plasma was chosen as a biological matrix in the proposed research as it is the most widely adopted biological fluid in drug discovery and clinical development. Furthermore, regulatory agencies (USFDA[16-18] and EMA[19-21]) also recommended using plasma as an appropriate biological matrix to measure selected drugs.

The following research aim and objectives were formulated in support of the facts as described above:

3.2 Aim

Method development, validation and *in-vivo* bioequivalence studies of novel oral anti-coagulants (NOACs) – dabigatran, rivaroxaban, and apixaban.

3.3 Objectives

The objective of the work was to develop and validate liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for determination of free (non-conjugated) dabigatran and total dabigatran (non-conjugated plus conjugated dabigatran after complete alkaline cleavage of dabigatran glucuronides), rivaroxaban, and apixaban in human plasma while adopting the latest regulatory norms and their applications to *in-vivo* bioequivalence studies.

While fulfilling the aforementioned objectives, it was decided for establishment of concerned methods to have the merits of high analytical sensitivity (lower limit of quantitation), selectivity (insignificant interference of matrix components), speed (short analytical run time per sample) and cost effectiveness.

Chapter 4 Method development, validation and *in-vivo* bioequivalence study of dabigatran

The objective was to develop and validate LC-MS/MS method for determination of free (non-conjugated) dabigatran and total dabigatran (non-conjugated plus conjugated dabigatran after complete alkaline cleavage of dabigatran glucuronides) in human plasma and their applications to *in-vivo* bioequivalence study.

4.1 Method development

When used in conjunction with HPLC, the ESI source provides ionization that is sensitive, robust, and reliable for quantification of lower concentrations in micro-litre sample volumes, as well as the ability to analyze both small and large molecules of diverse polarity in a complex biological matrix, making it the ionization source of choice for analysis. The analytes' and IS' signal intensities in human plasma were found to be adequate and the regression curves were linear. In full scan spectra, the analytes and IS formed primarily protonated quasi molecular ions [M+H] +, with m/z 472 for dabigatran and m/z 476 for the IS. Full scan product-ion spectra of the analytes and IS were investigated to determine dabigatran using SRM mode. The most abundant ion in the product-ion mass for dabigatran was at ≈ 289 and ≈ 172, and for IS it was ≈ 293. Collision activity of $[M+H]^+$ of these compounds was highly influenced by collision energy, with changes in collision energy resulting in significant changes in fragmentation processes. The MS state file parameters were tweaked to achieve the highest possible product ion strength. To achieve optimum sensitivity, the SRM transitions of m/z 472/289,172 for dabigatran and m/z 476/293 for IS were chosen.

Solid phase extraction (SPE) technique with different cartridge: Strata X, Hypurit HLB, and Plexa bond elute and various washing trials with acetonitrile, water, methanol, and formic acid were attempted. Optimization was done to minimize interferences at the retention times of analyte and IS. Trials were taken to achieve consistent recovery. The samples prepared using Strata-X 33 µm, polymeric reversed phase (30 mg/mL) SPE cartridges (Phenomenex), 20% methanol as washing solution and mixture of acetonitrile, methanol and buffer solution in the volume ratio of 30:20:50 as eluent yielded excellent results, consistent recovery and cleanliness with no compromises.

Since the total dabigatran is (non-conjugated plus conjugated dabigatran after complete cleavage of dabigatran glucuronides). Therefore for alkaline cleavage of dabigatran glucuronides, an incubation step was added to the free dabigatran extraction procedure while keeping the rest of the steps identical. Alkaline hydrolysis was performed using sodium hydroxide and potassium hydroxide with different buffer concentration and volume, different incubation temperature and time and post-incubation steps with different acidification conditions. Plasma sample treated with 100 µL of 0.3 Mol potassium hydroxide (for alkaline cleavage), incubated at 30°C for 2 hrs and acidified with 0.6% formic acid; yielded excellent results, consistent recovery and cleanliness with no compromises.

The retention time window between 0.70 to 1.70 min was finely achieved with negligible matrix effect. Thus matrix effects can be eliminated using Strata-X for a robust reliable method that is inherently consistent.

Following the physicochemical properties of dabigatran i.e. solubility, pKa and stability, performed several trials with different compositions of aqueous-organic components and mobile phase buffer solutions (ammonium acetate, ammonium formate, ammonium tri-fluoro acetate and ammonia/basified buffers) to determine the mobile phase suitability for optimum chromatography and sensitivity. Fine tuned mobile phase with methanol to optimize the retention time selectivity for control of matrix effect during chromatographic run. Found a mixture of acetonitrile, methanol and buffer solution (5 mM ammonium formate) in the volume ratio of 30:20:50 (v/v/v) as an appropriate mobile phase. Keeping the chromatographic run time of 2.5 min with retention time of 1.2 min for dabigatran and IS, confirmed the absence of matrix effect / carryover effect / any late eluting peaks by running the extended run of 4 times the regular run time and monitoring baseline.

Liquid chromatographic and mass spectrometric conditions

Analytes were separated on a Peerless basic C8, 150 mm x 4.6 mm, 5µm, HPLC column (Chromatopak) maintained at column oven temperature of 30^{o}C. An isocratic flow-rate of 1.0 mL/min with mobile phase acetonitrile: methanol: 5 mM ammonium formate buffer (30:20:50, v/v/v) was used for chromatographic separation with 20 µL injection volume. Analyte and internal standard were retained at ~1.15 min with total run time of 2.5 min.

A mixture of methanol and Milli-Q water in the volume ratio of 20:80 was used as needle wash solution to avoid carry over between two injections. Mass spectrometric detection was performed on an Applied Biosystems Sciex API 3200 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) interface. ESI ionization or Turbo ion spray was performed in the positive ion mode. The selected reaction monitoring mode (SRM) was attained per unit resolution in tandem mass spectrometer. Sum of multiple ions with precursor-product ion pairs of *m/z* 472/289 & 472/172 used to achieve optimum sensitivity of dabigatran and ion pair of m/z 476/293 used for dabigatran-D_4 with scan time (dwell time) for each transition 300 msec. The optimized MS state file source parameters for acquisition method were ion source temperature, 600°C; Ion spray voltage, 3500 V, Curtain gas (UHP nitrogen), 45 psi; Nebulizer Gas (UHP nitrogen), 75 psi; Auxiliary (turbo) Gas, 75 psi; and CAD gas, 3 psi.

The concentration of the unknown samples was calculated using linear regression analysis of spiked calibration standards with the reciprocal of the square of ratio of the analyte concentration to internal standard concentration as a weighting factor $1/(\text{concentration ratio})^2$.

4.2 Method validation

Method Information	
Extraction technique	Solid phase extraction (SPE)
Linearity	Free dabigatran: 1.04 - 403.11 ng/mL Total dabigatran: 1.04 to 406.49 ng/mL
Quality control (QC) samples levels (ng/mL)	Free dabigatran: LLOQ QC: 1.07, LQC: 2.67, MQC: 161.12 and HQC: 320.99 Total dabigatran: LLOQ QC: 1.05, LQC: 2.86, MQC: 160.33 and HQC: 320.66
Equipment	LC-MS/MS system coupled with Shimadzu Prominence UFLC system and API-3200 mass spectrometer
Software	Analyst software (version 1.6.3)
Column	Peerless basic C8, 150 mm x 4.6 mm, 5µm
Flow rate	1.0 mL/min
Rinsing volume	800 µL
Rinse mode	Before and after aspiration
Detection	Positive ion mode

Method Information	
Ion-source	Turbo ion spray
Column oven temperature	30°C
Sample cooler temperature	6°C
Injection volume	20 µL
Run time	2.5 min
Retention time	1.2 min
Mobile phase	Acetonitrile: 5 mM ammonium formate: Methanol and 0.2% formic acid (30:20:50, v/v/v)
Rinsing solution	50% acetonitrile in water (50:50)

4.3 Results and discussions

Method validation results are reported in the tables below:

Parameter	Results
System suitability	Precision for RTs and peak area response of analyte and IS was <5% for all system suitability tests.
Linearity	Linearity was established for a range of concentrations 1.04 - 403.11 ng/mL for free dabigatran and 1.04 - 406.49 ng/mL for total dabigatran with a regression factor ≥ 0.99.
Precision and accuracy	The method is accurate and precise at all concentration levels with reproducible results.
Sensitivity	The LLOQ was identifiable and reproducible at 1.04 ng/mL with precision 2.33 - 4.11% and accuracy 98.88 - 106.41% for free dabigatran and with precision 3.28 - 5.86% and accuracy 99.24 - 108.25% for total dabigatran. Signal-to-noise ratio (S/N) >26 in all estimated LLOQ samples.
Selectivity	The method has the ability to measure the analyte unequivocally in the presence of other compounds in the matrix with reproducible results.
Matrix effect	The %CV of the IS-normalized MF calculated from six different lots of matrix was precise at three concentration levels (LQC, MQC and HQC).
Dilution integrity	Dilution integrity was reproducible at 2-fold and 4-fold dilution with a precision of 2.14% and 1.80% (for free dabigatran) and 1.26% and 2.31% (for total dabigatran) respectively.
Ruggedness	The capacity of the method remained unaffected with precision

Parameter	Results
	≤0.81% and accuracy 97.83 - 101.56% (for free dabigatran) and precision ≤1.89% and accuracy 93.36 - 95.02% (for total dabigatran).

Recovery			
Global % recovery (%CV)	**Plain**	**Hemolysed**	**Lipemic**
Free dabigatran	83.31 (2.74)	86.26 (1.17)	86.85 (0.81)
Total dabigatran	93.66 (1.49)	89.11 (3.07)	93.73 (2.25)
Dabigatran D_4	93.49 (1.38)	91.64 (2.14)	94.50 (1.28)

Matrix effect (IS normalized matrix factor)						
QC Sample	**Free dabigatran - Mean (%CV)**			**Total dabigatran - Mean (%CV)**		
	Plain	Hemolysed	Lipemic	Plain	Hemolysed	Lipemic
LQC	0.99 (0.81)	0.99 (2.62)	0.99 (1.81)	1.02 (2.04)	1.00 (3.21)	1.02 (2.67)
MQC	1.01 (1.85)	1.00 (0.37)	1.01 (0.39)	1.00 (1.99)	0.99 (1.61)	0.99 (2.03)
HQC	1.02 (1.10)	1.01 (0.83)	1.01 (0.67)	1.01(0.84)	1.01 (1.31)	1.02 (1.29)
Global	1.01 (1.68)	1.00 (1.56)	1.00 (1.62)	1.01 (1.77)	1.00 (2.34)	1.01 (2.41)

Precision and Accuracy				
	Free dabigatran		**Total dabigatran**	
	Precision (%CV)	Accuracy (%)	Precision (%CV)	Accuracy (%)
Inter-day	1.21 – 3.86	96.1 – 101.74	1.97 – 4.42	103.94 – 109.98
Intra-day	1.18 – 3.81	96.02 – 100.28	1.56 – 3.17	103.11 – 111.15
Within batch	0.74 – 2.61	95.51 – 104.67	0.74 – 5.03	102.73 – 112.50

Re-injection reproducibility	**% nominal (CV)**
LQC	103.3 (1.38)
HQC	98.0 (0.95)
Production batch analysis and positional difference check	
Precision	0.53 – 2.01%
Accuracy	94.12 – 96.32%

Stability studies

The stability tests were intended to cover the possible conditions that samples may undergo. Various stability studies such as: bench top stability (21 h for free and 6 h for total dabigatran), auto-sampler stability (49 h for free and 27 h for total dabigatran), freeze thaw stability (4 cycles for free and 5 cycles for total dabigatran), post

preparative bench top stability (24 h for free and 26 h for total dabigatran), blood stability (90 min), refrigerated stock solution stability between 2-8°C (42 days 19 h), long-term stability (at -75°C for 96 days), and long-term stability (at -20°C for 96 days) were conducted to ensure that every step taken during sample preparation and analysis, in addition to the storage conditions applied do not alter the results.

The% stability was measured as% accuracy of the results. The consistency in results fulfilled the stability requirements.

Conclusion

LC-MS/MS method for the estimation of free (non-conjugated) dabigatran and total dabigatran (non-conjugated plus conjugated dabigatran after complete alkaline cleavage of dabigatran glucuronides) from human plasma containing sodium citrate as anticoagulant using dabigatran D_4 as an internal standard was developed and validated with reproducible results. This method had a limit of reliable quantification of 1.04 ng/mL.

4.4 Bioequivalence study of dabigatran

Bioequivalence study was conducted to evaluate the comparative oral bioavailability of the test formulation; Dabigatran etexilate mesylate Capsules 150 mg (Macleods) with reference formulation; Pradaxa (dabigatran etexilate) 150 mg hard Capsules (Boehringer Ingelheim International GmbH, Germany) under fasting condition in healthy adult, human subjects.

Prior approval was taken from the IEC (Independent Ethics Committee) and written informed consent was obtained from the study participants.

The study was single center, open label, randomized, fasting, single oral dose, two-treatment, two-sequence and two-way crossover study with a washout interval of seven (7) days between dosing.

Eighteen (18) healthy human male subjects age between 18-45 years body mass index (BMI) range 18.50-30.00 kg/m^2 with body weight ≥50 kg, with normal prothrombin time (PT), activated partial thromboplastin time (aPTT), and creatinine clearance (CrCl) value >50 mL/min and meeting the pre-defined inclusion criteria and none of the defined exclusion criteria were enrolled in the study. During each period, after an overnight fast of at least 10 h, the study product (1 × 150 mg capsule) of reference or test was administered orally with 240 mL of tepid water as per the randomization schedule.

During each period of the study, 24 (each of 4 mL) pharmacokinetic blood samples were collected via an indwelling cannula into a tube containing sodium citrate solution (0.445 mL of 0.105 mol/L) as anticoagulant according to the following schedule: pre-dose and at 0.17, 0.33, 0.50, 0.67, 0.83, 1.00, 1.17, 1.33, 1.50, 1.75, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 6.00, 8.00, 12.00, 18.00, 24.00, 36.00 and 48.00 h post-dose. Post-blood collection, samples were kept cool by placing in ice bath and centrifuged at 4000 rpm for 10 min between 4 to 8°C. Supernatant plasma was aliquoted into pre-labeled polypropylene tubes and stored in freezer set at -75°C until analysis.

4.5 Results and discussions

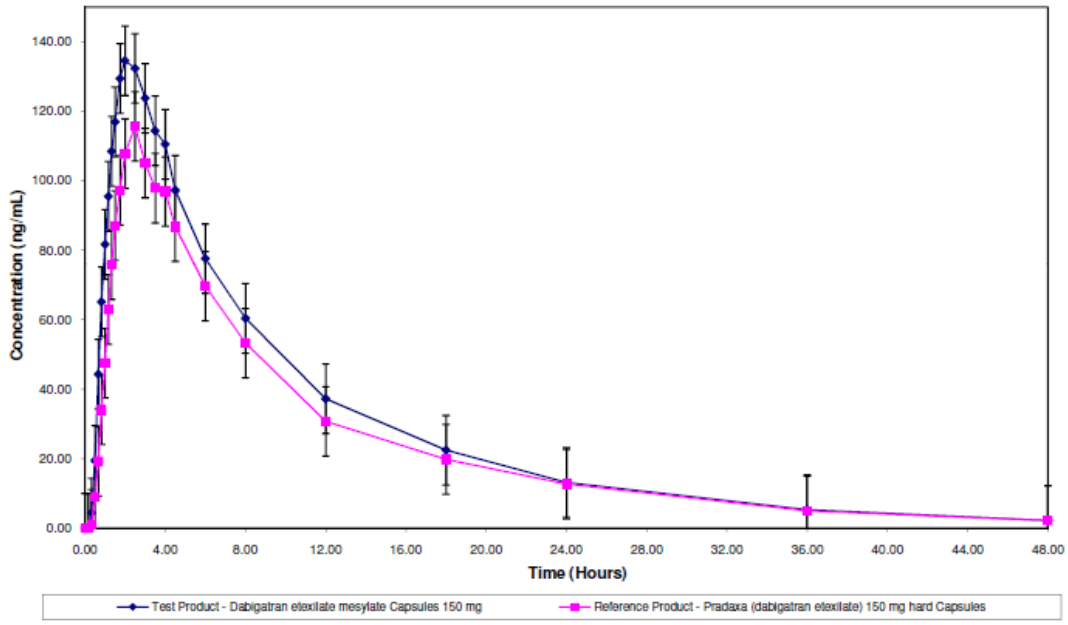
The plasma samples of sixteen (16) subjects completing the study were analyzed following validated method as described to obtain the drug concentrations for both dabigatran (non-conjugated or free form) and total dabigatran (non-conjugated and dabigatran glucuronide) and the data were taken for pharmacokinetic (PK) and statistical evaluations.

To demonstrate reproducibility and verify the reliability of the reported study sample analyte concentrations, incurred sample reanalysis (ISR) was performed in a separate batch by randomly selecting subject samples, including samples near C_{max} and the elimination phase in the PK profile of the analytes. The ISR experiment carried out by re-analyzing 128 incurred samples of study for free and total dabigatran. Reanalysis values of 100% (128 out of 128) samples of free dabigatran and 99.2% (127 out of 128) samples of total dabigatran were within acceptance range of ± 20% of original values.

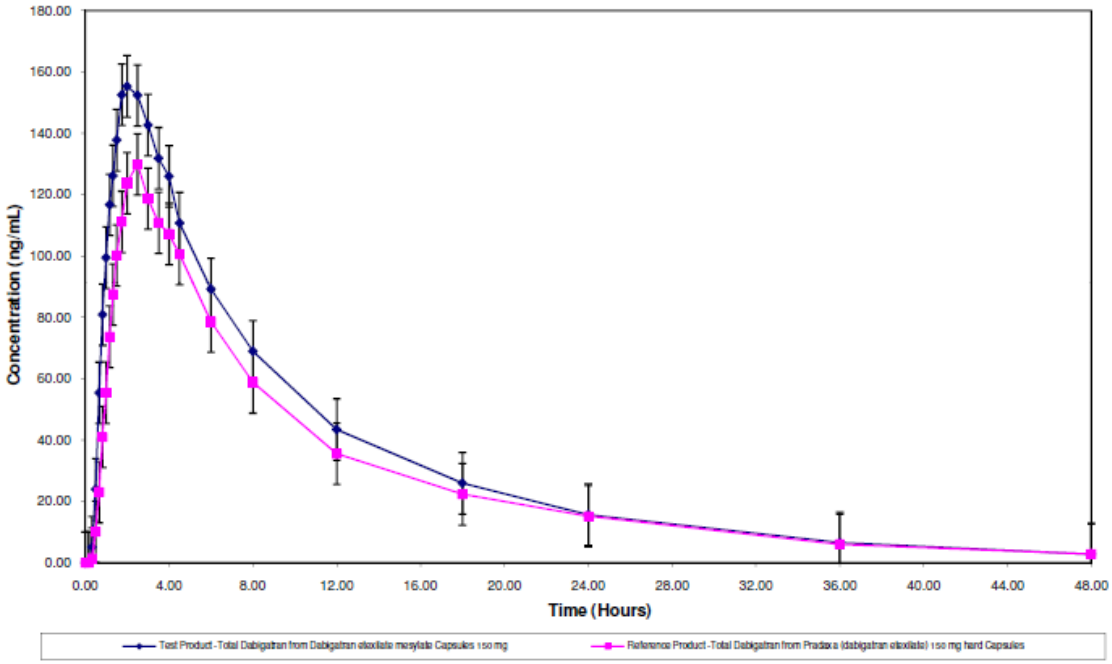
The PK parameters were calculated by non-compartmental methods using statistical software, SAS® version 9.4. The summary statistics for mean PK parameters are summarized in the below table.

Statistical summary of the comparative bioavailability data (N=16)							
Pharmacokinetic Parameters	**Geometric Mean**		**Ratio (T/R) (%)**	**Intra Subject C.V. (%)**	**Power (%)**	**90% Confidence Interval (%)**	
	Reference	**Test**				**Lower**	**Upper**
Free Dabigatran							
C_{max}	114.992	137.220	119.33	43.37	27.12	92.15	154.53
AUC_{0-t}	1015.132	1228.870	121.06	39.76	31.33	95.36	153.67
Total Dabigatran							
C_{max}	129.553	157.334	121.44	45.71	24.90	92.59	159.28
AUC_{0-t}	1175.245	1439.818	122.51	39.77	31.32	96.50	155.53

The mean plasma concentration *versus* time curves for free and total dabigatran is illustrated in below figures.



Comparative linear plot of free dabigatran mean plasma concentration (N=16)



Comparative linear plot of total dabigatran mean plasma concentration (N=16)

Chapter 5 Method development, validation and *in-vivo* bioequivalence study of rivaroxaban

The objective was to develop and validate LC-MS/MS method for determination rivaroxaban in human plasma and their applications to *in-vivo* bioequivalence study.

5.1 Method development

When used in conjunction with HPLC, the ESI source provides ionization that is sensitive, robust, and reliable for quantification of lower concentrations in micro-litre sample volumes, as well as the ability to analyze both small and large molecules of diverse polarity in a complex biological matrix, making it the ionization source of choice for analysis. The analytes' and IS' signal intensities in human plasma were found to be adequate and the regression curves were linear. In full scan spectra, the analytes and IS formed primarily protonated quasi molecular ions [M+H] +, with m/z 436 for rivaroxaban and m/z 440 for the IS. Full scan product-ion spectra of the analytes and IS were investigated to determine rivaroxaban using SRM mode. The most abundant ion in the product-ion mass for rivaroxaban was at ≈ 145 for rivaroxaban and IS. Collision activity of $[M+H]^+$ of these compounds was highly influenced by collision energy, with changes in collision energy resulting in significant changes in fragmentation processes. The MS state file parameters were tweaked to achieve the highest possible product ion strength. To achieve optimum sensitivity, the SRM transitions of m/z 436/145 for rivaroxaban and *m*/z 440/145 for IS were chosen.

Solid phase extraction (SPE) technique with different cartridge: Strata X, Hypurit HLB, and Plexa bond elute and various washing trials with acetonitrile, water, methanol, and formic acid were attempted. Optimization was done to minimize interferences at the retention times of analyte and IS. Trials were taken to achieve consistent recovery. The samples prepared using Plexa bond elute 33 µm, polymeric reversed phase (30 mg/mL) SPE cartridges (Agilent), 5% acetonitrile as washing solution and 80% acetonitrile as eluent yielded excellent results, consistent recovery and cleanliness with no compromises. The retention time 1.18 min was finely achieved with negligible matrix effect. Thus matrix effects can be eliminated using Plexa bond elute for a robust reliable method that is inherently consistent.

Following the physicochemical properties of rivaroxaban i.e. solubility, pKa and stability, performed several trials with different compositions of aqueous-organic

components and mobile phase buffer solutions (ammonium acetate and acetonitrile) to determine the mobile phase suitability for optimum chromatography and sensitivity. Fine tuned mobile phase with methanol to optimize the retention time selectivity for control of matrix effect during chromatographic run. Found a mixture of acetonitrile, and buffer solution (5 mM ammonium acetate) in the volume ratio of 80:20 (v/v) as an appropriate mobile phase. Each chromatographic run was completed within 1.8 minutes and elution time was 1.18 minutes for rivaroxaban and IS.

Liquid chromatographic and mass spectrometric conditions

Analytes were separated on a Gemini C18, 150 mm x 4.6 mm, 5µm, HPLC column (Phenomenex) maintained at column oven temperature of 40^{o}C. An isocratic flow-rate of 1.5 mL/min with mobile phase acetonitrile: 5 mM ammonium acetate buffer (80:20 v/v) was used for chromatographic separation with 5 µL injection volume. Analyte and internal standard were retained at ~1.18 min with total run time of 1.8 min.

A mixture of acetonitrile and Milli-Q water in the volume ratio of 50:50 was used as needle wash solution to avoid carry over between two injections. Mass spectrometric detection was performed on an Applied Biosystems Sciex API 4000 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) interface. ESI ionization or Turbo ion spray was performed in the positive ion mode. The selected reaction monitoring mode (SRM) was attained per unit resolution in tandem mass spectrometer. Sum of multiple ions with precursor-product ion pairs of *m/z* 436/145 used to achieve optimum sensitivity of Rivaroxaban and ion pair of m/z 440/145 used for Rivaroxaban D_4 with scan time (dwell time) for each transition 250 msec. The optimized MS state file source parameters for acquisition method were ion source temperature, 600°C; Ion spray voltage, 5500 V, Curtain gas (UHP nitrogen), 40 psi; Nebulizer Gas (UHP nitrogen), 50 psi; Auxiliary (turbo) Gas, 60 psi; and CAD gas, 12 psi.

The concentration of the unknown samples was calculated using linear regression analysis of spiked calibration standards with the reciprocal of the square of ratio of the analyte concentration to internal standard concentration as a weighting factor 1/(concentration ratio)^2.

5.2 Method validation

Method Information	
Extraction technique	Solid phase extraction (SPE)
Linearity	5.96 - 800.73 ng/mL
Quality control (QC) samples levels (ng/mL)	LLOQ QC: 5.98, LQC: 16.00, MQC-A: 75.37, MQC: 256.39 and HQC: 656.47
Equipment	LC-MS/MS system coupled with Shimadzu Prominence UFLC system and API-4000 mass spectrometer
Software	Analyst software (version 1.6.3)
Column	Gemini C18 (150x4.6) mm, 5µm
Flow rate	1.5 mL/min
Rinsing volume	500 µL
Rinse mode	Before and after aspiration
Detection	Positive ion mode
Ion-source	Turbo ion spray
Column oven temperature	40°C
Sample cooler temperature	4°C
Injection volume	5 µL
Run time	1.80 min
Retention time	1.18 min
Mobile phase	80:20 (v/v)- acetonitrile and buffer solution (5 mM ammonium acetate)
Rinsing solution	50% acetonitrile in water (50:50)

5.3 Results and discussion

Method validation results are reported in the tables below:

Parameter	**Results**
System suitability	Precision for RTs and peak area response of analyte and IS was <5% for all system suitability tests.
Linearity	Linearity was established for a range of concentrations 5.96 - 800.73 ng/mL with a regression factor ≥ 0.99.
Precision and accuracy	The method is accurate and precise at all concentration levels with reproducible results.
Sensitivity	The LLOQ was identifiable and reproducible at 5.96 ng/mL with precision 4.66% and accuracy 97.4%. Signal-to-noise ratio (S/N) ≥32 in all estimated LLOQ samples.
Selectivity	The method has the ability to measure the analyte unequivocally

Parameter	Results
	in the presence of other compounds in the matrix with reproducible results.
Matrix effect	The %CV of the IS-normalized MF calculated from six different lots of matrix was precise at three concentration levels (LQC, MQC and HQC).
Dilution integrity	Dilution integrity was reproducible at 2-fold and 4-fold dilution with a precision of 2.06% and 0.77% respectively.
Ruggedness	The capacity of the method remained unaffected with precision ≤4.00% and accuracy 97.3 – 101.

Recovery			
Global % recovery (%CV)	**Plain**	**Hemolysed**	**Lipemic**
Rivaroxaban	91.7 (1.07)	91.6 (1.77)	88.9 (0.53)
Rivaroxaban D_4	91.2 (2.61)	91.9 (1.30)	89.2 (1.00)

Matrix effect (IS normalized matrix factor) - Mean (%CV)			
QC Sample	**Plain**	**Hemolysed**	**Lipemic**
LQC	1.00 (2.26)	1.00 (2.33)	1.01 (1.67)
MQC	1.00 (0.71)	1.01 (1.00)	1.01 (1.12)
HQC	1.01 (0.87)	1.01 (1.36)	1.01 (1.22)
Global	1.00 (1.39)	1.01 (1.63)	1.01 (1.29)

Precision and accuracy		
	Precision (%CV)	**Accuracy (%)**
Inter-day	1.08 – 3.75	96.3 – 102
Intra-day	0.96 – 2.61	94.7 – 102
Within batch	0.68 – 4.08	94.5 – 103

Re-injection reproducibility	
% Nominal (LQC)	99.2 – 107%
% Nominal (HQC)	96.1 – 102%
Production batch analysis and positional difference check	
Precision	≤ 2.87%
Accuracy	95.5 – 104%

Stability studies

The stability tests were intended to cover the possible conditions that samples may undergo. Various stability studies such as: bench top stability (17 h), auto-sampler stability (75 h), freeze thaw stability (5 cycles), dry ice stability (3 h), post preparative bench top stability (26 h), blood stability (90 min), short term bench top stock solution stability at room temperature (24 h), refrigerated stock solution stability between 2-8°C (5 days 22 h), spiking dilution bench top stability at room temperature (24 h), spiking dilution refrigerator stability between 2-8°C (5 Days 22 hrs) and long-term stability (at -75°C for 92 days) were conducted to ensure that every step taken during sample preparation and analysis, in addition to the storage conditions applied do not alter the results.

The% stability was measured as% accuracy of the results. The consistency in results fulfilled the stability requirements.

Conclusion

LC-MS/MS method for the estimation of rivaroxaban from human plasma containing K_2EDTA as anticoagulant using rivaroxaban D_4 as an internal standard was developed and validated with reproducible results. This method had a limit of reliable quantification of 5.96 ng/mL.

5.4 Bioequivalence study of rivaroxaban

Bioequivalence study was conducted to evaluate the comparative oral bioavailability of the test formulation; Rivaroxaban film coated tablets 20 mg (Macleods) with reference formulation; XARELTO® (rivaroxaban) Tablets 20 mg (Janssen Pharmaceuticals Inc., USA) under fasting condition in healthy adult, human subjects.

Prior approval was taken from the IEC and written informed consent was obtained from the study participants.

The study was single center, open label, randomized, fasting, single oral dose, two-treatment, four-period, two-sequence and fully replicated crossover study with a washout interval of seven (7) days between dosing.

Forty (40) healthy human male subjects age between 18-45 years body mass index (BMI) range 18.50-30.00 kg/m^2 with body weight ≥50 kg, with normal prothrombin time (PT), activated partial thromboplastin time (aPTT), and creatinine clearance

(CrCl) value >50 mL/min and meeting the pre-defined inclusion criteria and none of the defined exclusion criteria were enrolled in the study.

During each period, after an overnight fast of at least 10 h, the study product (1 × 20 mg tablet) of reference or test was administered orally with 240 mL of tepid water as per the randomization schedule.

During each period of the study, 24 (each of 3 mL) pharmacokinetic blood samples were collected via an indwelling cannula into a tube containing K_2EDTA as anticoagulant according to the following schedule: pre-dose and at 0.25, 0.50, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 8.00, 10.00, 14.00, 18.00, 24.00, 30.00, 36.00 and 48.00 h post-dose. Post-blood collection, samples were centrifuged at 4000 rpm for 10 min between 4 to 8°C. Supernatant plasma was aliquoted into pre-labeled polypropylene tubes and stored in freezer set at -75°C until analysis.

5.5 Results and discussions

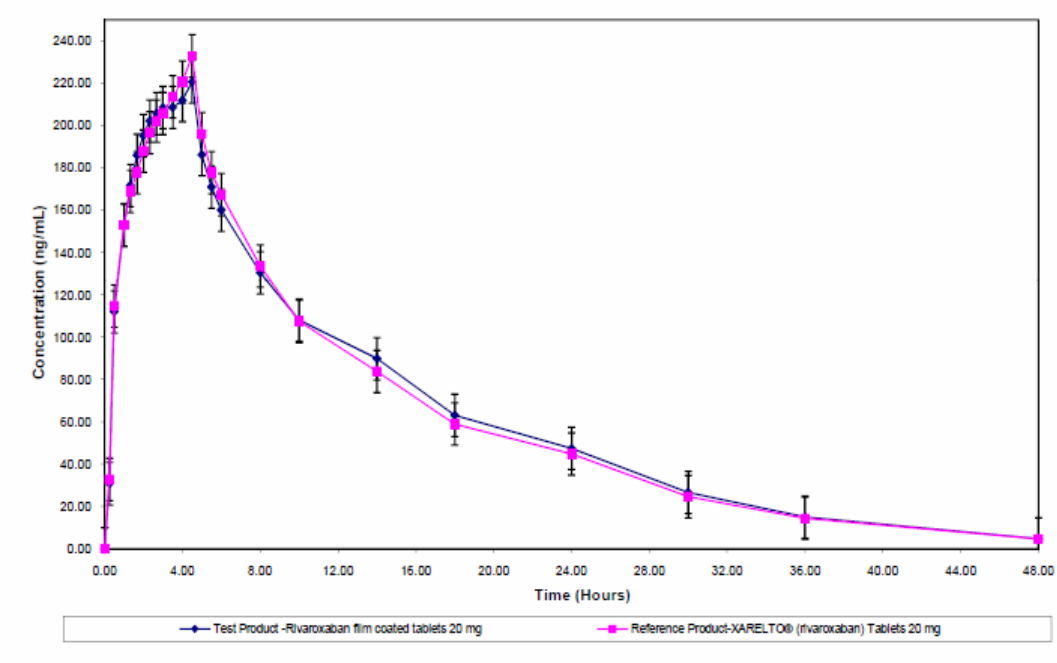
The plasma samples of thirty-seven (37) subjects completing the study were analyzed following validated method as described to obtain the drug concentrations for rivaroxaban and the data were taken for pharmacokinetic (PK) and statistical evaluations.

Incurred sample reanalysis (ISR) was performed in a separate batch by randomly selecting 8.34% of total subject samples, including samples near C_{max} and the elimination phase in the PK profile of the analytes. The ISR experiment carried out by re-analyzing 300 incurred samples of study. Reanalysis values of 98.3% (295 out of 300) samples were within acceptance range of ± 20% of original values.

The PK parameters were calculated by non-compartmental methods using statistical software, SAS® version 9.4. The summary statistics for mean PK parameters are summarized in the below table.

Statistical summary of the comparative bioavailability data [n=74 (37 subjects)]							
PK parameters	Geometric mean		Ratio (T/R) (%)	Power (%)	90% CI (%)	Ratio (S_{WT}/S_{WR})	90% Upper CI
	Reference	Test					
C_{max} (ng/mL)	247	235	95.1	100	89.24 - 101.33	1.21	1.6
AUC_{0-t} (ng*hrs/mL)	2917	2953	101	100	96.82 - 105.83	1.01	1.3
$AUC_{0-\infty}$ (ng*hrs/mL)	3055	3078	101	100	96.48 - 105.83	0.98	1.3

The mean plasma concentration *versus* time curves for rivaroxaban is illustrated in below figure:



Comparative linear plot of rivaroxaban mean plasma concentration (N=74)

Chapter 6 Method development, validation and *in-vivo* bioequivalence study of apixaban

The objective was to develop and validate LC-MS/MS method for determination apixaban in human plasma and their applications to *in-vivo* bioequivalence study.

6.1 Method development

When used in conjunction with HPLC, the ESI source provides ionization that is sensitive, robust, and reliable for quantification of lower concentrations in micro-litre sample volumes, as well as the ability to analyze both small and large molecules of diverse polarity in a complex biological matrix, making it the ionization source of choice for analysis. The analytes' and IS' signal intensities in human plasma were found to be adequate and the regression curves were linear. In full scan spectra, the analytes and IS formed primarily protonated quasi molecular ions [M+H] +, with m/z 460 for apixaban and m/z 464 for the IS. Full scan product-ion spectra of the analytes and IS were investigated to determine apixaban using SRM mode. The most abundant ion in the product-ion mass for apixaban was at 443 for apixaban and 447 for IS. Collision activity of $[M+H]^+$ of these compounds was highly influenced by collision energy, with changes in collision energy resulting in significant changes in fragmentation processes. The MS state file parameters were tweaked to achieve the highest possible product ion strength. To achieve optimum sensitivity, the SRM transitions of m/z 460/443 for apixaban and *m*/z 464/447 for IS were chosen.

Solid phase extraction (SPE) technique with different cartridge: Strata X, Oasis HLB, Hypurit HLB, and Plexa bond elute and various washing trials with water, methanol, formic acid and ammonia were attempted. Optimization was done to minimize interferences at the retention times of analyte and IS. Trials were taken to achieve consistent recovery. The samples prepared using Strata-X 33 µm, polymeric reversed phase (30 mg/mL) SPE cartridges (Phenomenex), 10% methanol as washing solution and methanol as eluent yielded excellent results, consistent recovery and cleanliness with no compromises. The retention time 0.9 min was finely achieved with negligible matrix effect. Thus matrix effects can be eliminated using Strata-X for a robust reliable method that is inherently consistent.

Following the physicochemical properties of apixaban i.e. solubility, pKa and stability, performed several trials with different compositions of aqueous-organic

components and mobile phase buffer solutions (ammonium formate and acetonitrile) to determine the mobile phase suitability for optimum chromatography and sensitivity. Fine tuned mobile phase with methanol to optimize the retention time selectivity for control of matrix effect during chromatographic run. Found a mixture of acetonitrile, and buffer solution (2 mM ammonium formate) in the volume ratio of 50:50 (v/v) as an appropriate mobile phase. Each chromatographic run was completed within 1.8 minutes and elution time was 0.9 minutes for apixaban and IS.

Liquid chromatographic and mass spectrometric conditions

Analytes were separated on a Gemini C18, 50 mm x 4.6 mm, 3µm, HPLC column (Phenomenex) maintained at column oven temperature of 40^{o}C. An isocratic flow-rate of 1.0 mL/min with mobile phase acetonitrile: 2 mM ammonium formate buffer (50:50 v/v) was used for chromatographic separation with 5 µL injection volume. Analyte and internal standard were retained at ~0.9 min with total run time of 1.8 min. A mixture of acetonitrile and Milli-Q water in the volume ratio of 50:50 was used as needle wash solution to avoid carry over between two injections. Mass spectrometric detection was performed on an Applied Biosystems Sciex API 4000 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) interface. ESI ionization or Turbo ion spray was performed in the positive ion mode. The selected reaction monitoring mode (SRM) was attained per unit resolution in tandem mass spectrometer. Sum of multiple ions with precursor-product ion pairs of *m/z* 460/443 used to achieve optimum sensitivity of Apixaban and ion pair of m/z 464/447 used for Apixaban $^{13}CD_3$ with scan time (dwell time) for each transition 250 msec. The optimized MS state file source parameters for acquisition method were ion source temperature, 600°C; Ion spray voltage, 2500 V, Curtain gas (UHP nitrogen), 30 psi; Nebulizer Gas (UHP nitrogen), 50 psi; Auxiliary (turbo) Gas, 70 psi; and CAD gas, 8 psi.

The concentration of the unknown samples was calculated using linear regression analysis of spiked calibration standards with the reciprocal of the square of ratio of the analyte concentration to internal standard concentration as a weighting factor 1/(concentration ratio)^2.

6.2 Method validation

Method Information	
Extraction technique	Solid phase extraction (SPE)
Linearity	1.01 - 280.16 ng/mL
Quality control (QC) samples levels (ng/mL)	LLOQ QC: 1.02, LQC: 2.80, MQC-A: 50.13, MQC: 98.02 and HQC: 238.01
Equipment	LC-MS/MS system coupled with Shimadzu Prominence UFLC system and API-4000 mass spectrometer
Software	Analyst software (version 1.5.1)
Column	Gemini C18 (50x4.6) mm, 3µm
Flow rate	1.000 mL/min
Rinsing volume	500 µL
Rinse mode	Before and after aspiration
Detection	Positive ion mode
Ion-source	Turbo ion spray
Column oven temperature	40°C
Sample cooler temperature	4°C
Injection volume	5.00 µL
Run time	1.80 minute
Retention time	Apixaban: ~0.90 min; Apixaban $^{13}CD_3$: ~ 0.89 min
Mobile phase	50:50 (v/v)- Acetonitrile and buffer solution (2 mM ammonium formate)
Rinsing solution	50% acetonitrile in water (50:50)

6.3 Results and discussion

Method validation results are reported in the tables below:

Parameter	**Results**
System suitability	Precision for RTs and peak area response of analyte and IS was <5% for all system suitability tests.
Linearity	Linearity was established for a range of concentrations 1.01 - 280.16 ng/mL with a regression factor ≥ 0.99.
Precision and accuracy	The method is accurate and precise at all concentration levels with reproducible results.
Sensitivity	The LLOQ was identifiable and reproducible at 1.01 ng/mL with

Parameter	Results
	precision 1.93 – 3.26% and accuracy 104 – 111%. Signal-to-noise ratio (S/N) >24 in all estimated LLOQ samples.
Selectivity	The method has the ability to measure the analyte unequivocally in the presence of other compounds in the matrix with reproducible results.
Matrix effect	The %CV of the IS-normalized MF calculated from six different lots of matrix was precise at three concentration levels (LQC, MQC and HQC).
Dilution integrity	Dilution integrity was reproducible at 2-fold and 4-fold dilution with a precision of 0.96% and 1.46% respectively.
Ruggedness	The capacity of the method remained unaffected with precision ≤4.87% and accuracy 98 – 103%.

Recovery			
Global % recovery (%CV)	**Plain**	**Hemolysed**	**Lipemic**
Apixaban	77.0 (5.36)	77.2 (3.90)	77.5 (4.14)
Apixaban $^{13}CD_3$	77.7 (3.50)	80.4 (5.06)	80.6 (4.55)

Matrix effect (IS normalized matrix factor) - Mean (%CV)			
QC Sample	**Plain**	**Hemolysed**	**Lipemic**
LQC	1.00 (2.18)	1.00 (1.55)	0.99 (1.30)
MQC	0.99 (1.46)	0.99 (1.66)	1.00 (1.55)
HQC	1.02 (1.57)	1.01 (0.7)	1.01 (0.85)
Global	1.00 (1.99)	1.00 (1.41)	1.00 (1.41)

Precision and accuracy		
	Precision (%CV)	**Accuracy (%)**
Inter-day	1.21 – 3.21	96.5 – 102
Intra-day	1.21 – 3.13	96.7 – 102
Within batch	1.18 – 4.14	95.9 – 104

Re-injection reproducibility	
% Nominal (LQC)	99 – 105%
% Nominal (HQC)	97.5 – 103%

Production batch analysis and positional difference check	
Precision	≤4.08%
Accuracy	97.2 – 102%

Stability studies

The stability tests were intended to cover the possible conditions that samples may undergo. Various stability studies such as: bench top stability (20 h), auto-sampler stability (74 h), evaporator stability (1 h), freeze thaw stability (5 cycles), dry ice stability (4 h), post preparative bench top stability (20 h), blood stability (90 min), short term bench top stock solution stability at room temperature (24 h), refrigerated stock solution stability below 12°C (7 days), spiking dilution bench top stability at room temperature (23 h) and long-term stability (at -75°C for 51 days) were conducted to ensure that every step taken during sample preparation and analysis, in addition to the storage conditions applied do not alter the results.

The% stability was measured as% accuracy of the results. The consistency in results fulfilled the stability requirements.

Conclusion

LC-MS/MS method for the estimation of apixaban from human plasma containing K_2EDTA as anticoagulant using apixaban $^{13}CD_3$ as an internal standard was developed and validated with reproducible results. This method had a limit of reliable quantification of 1.01 ng/mL.

6.4 Bioequivalence study of apixaban

Bioequivalence study was conducted to evaluate the comparative oral bioavailability of the test formulation; Apixaban Tablets 5 mg (Macleods) with reference formulation; Eliquis® (apixaban) film-coated Tablets 5 mg (Bristol-Myers Squibb, UK) under fasting condition in healthy adult, human subjects.

Prior approval was taken from the IEC and written informed consent was obtained from the study participants.

The study was single center, open label, randomized, fasting, single oral dose, two-treatment, two-sequence and two-way crossover study with a washout interval of seven (7) days between dosing.

Twenty-four (24) healthy human male subjects age between 18-45 years body mass index (BMI) range 18.50-30.00 kg/m^2 with body weight ≥50 kg, with normal

Prothrombin time/International Normalized Ratio (PT/INR) and meeting the pre-defined inclusion criteria and none of the defined exclusion criteria were enrolled in the study.

During each period, after an overnight fast of at least 10 h, the study product (1 × 5 mg tablet) of reference or test was administered orally with 240 mL of tepid water as per the randomization schedule.

During each period of the study, 26 (each of 5 mL) pharmacokinetic blood samples were collected via an indwelling cannula into a tube containing K_2EDTA as anticoagulant according to the following schedule: pre-dose and at 0.17, 0.33, 0.50, 1.00, 1.50, 2.00, 2.33, 2.67, 3.00, 3.25, 3.50, 3.75, 4.00, 4.33, 4.67, 5.00, 5.50, 6.00, 8.00, 12.00, 18.00, 24.00, 30.00, 36.00 and 48.00 h post-dose. Post-blood collection, samples were centrifuged at 4000 rpm for 10 min between 4 to 8°C. Supernatant plasma was aliquoted into pre-labeled polypropylene tubes and stored in freezer set at -75°C until analysis.

6.5 Results and discussion

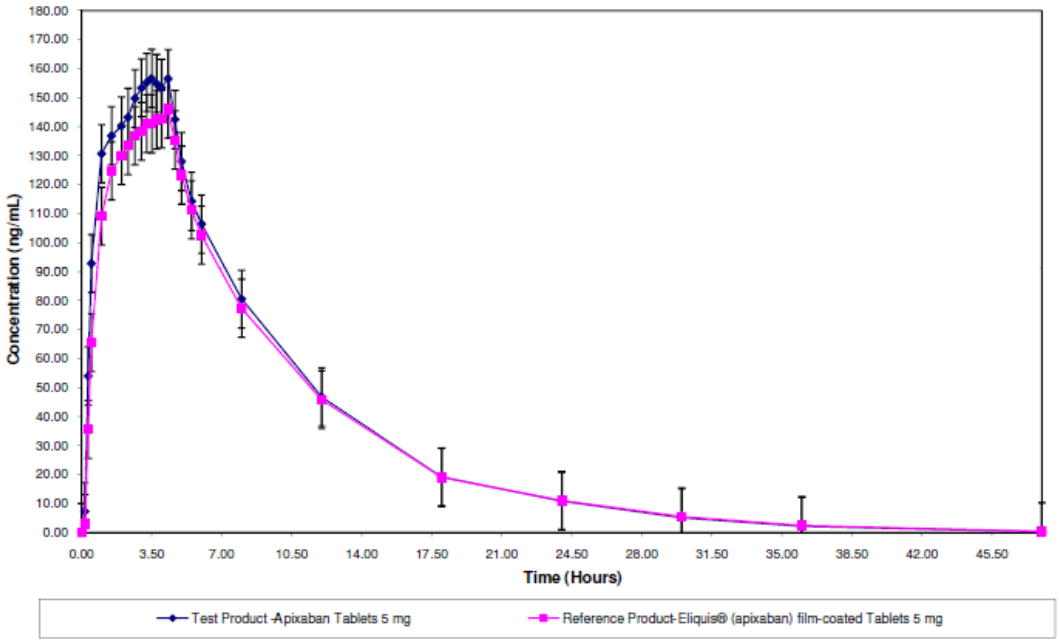
The plasma samples of twenty-three (23) subjects completing the study were analyzed following validated method as described to obtain the drug concentrations for apixaban and the data were taken for pharmacokinetic (PK) and statistical evaluations. Incurred sample reanalysis (ISR) was performed in a separate batch by randomly selecting ~10% of total subject samples, including samples near C_{max} and the elimination phase in the PK profile of the analytes. The ISR experiment carried out by re-analyzing 115 incurred samples of study. Reanalysis values of 99.1% (114 out of 115) samples were within acceptance range of ± 20% of original values.

The PK parameters were calculated by non-compartmental methods using statistical software, SAS® version 9.4. The summary statistics for mean PK parameters are summarized in the below table.

Statistical summary of the comparative bioavailability data (n=23)

Pharmacokinetic Parameters	Geometric Mean		Ratio (T/R) (%)	Intra Subject CV (%)	Power (%)	90% CI (%)	
	Reference	Test				Lower	Upper
C_{max} (ng/mL)	150	168	112	13.0	99.9	104.91	119.70
AUC_{0-t} (ng.h/mL)	1432	1545	108	9.54	100	102.82	113.25

The mean plasma concentration *versus* time curves for apixaban is illustrated in below figure.



Comparative linear plot of apixaban mean plasma concentration (N=23)

Chapter 7 Summary and conclusion

To the best of our knowledge, the current LC-MS/MS bioanalytical method for quantification of selected NOACs in human plasma has been reported with adequate information in accordance with the most recent regulatory criteria.

The main goal of this study's chromatographic condition optimization was to produce good resolution and symmetric peak morphologies of the analyte and IS while using isocratic elution and a reduced sample run time. The use of selected column in UHPLC improves separation throughput and efficiency while lowering retention time and solvent volume required during separations. The SPE technique was employed as an extraction method improvement strategy in these works to improve plasma sample clean-up and reduce matrix effect, allowing for superior method performance. With the selected limit of quantification and stable labeled IS, the presented approach showed high specificity and linearity, and the findings obtained after subject analysis indicated that the LOQ values selected are sufficient to provide data for computation of the requisite pharmacokinetic data and demonstrate bioequivalence. The approach is very selective, as evidenced by the matrix effect data. The isocratic method allows for a rapid turnaround time to deal with the high demand from clinical pharmacokinetic research. The outcomes of the incurred sample re-analysis indicated strong assay accuracy of the target analyte in their actual matrix in the experimental conditions used.

The current bioanalysis methods for the estimation of NOACs are presented with a fast, easy, sensitive, and accurate determination from human plasma with SPE using LC-MS/MS. The method described does not necessitate costly chemicals and solvents and does not occupy complex instrumentation or difficult sample preparation. The present work representing the accurate, reproducibility in data as well as in extraction recoveries, reliable, and simple method with a minimum run time that is required for a large number of samples to analyze. Methods tested with various parameters like linearity, selectivity, precision, accuracy, stability studies; extended batch analysis, carry over, matrix effect, sensitivity and recovery thus found to be supported to pharmacokinetic, bioavailability and bioequivalence studies of NOACs. The proposed methods were furthermore applied to the pharmacokinetic analysis and ensured the successful application.

The new approach with complete validity has the potential to make a substantial contribution to the further advancement of the regulatory submission of clinical research programs and drug development of NOACs.

Publications

[1] Shaikh K, Mungantiwar A, Halde S, Pandita N. Liquid chromatography–tandem mass spectrometry method for determination of rivaroxaban in human plasma and its application to a pharmacokinetic study. Eur J Mass Spectrom 2020; 26(2):91-105.

[2] Shaikh K, Mungantiwar A, Halde S, Pandita N. A liquid chromatography-tandem mass spectrometry method for the determination of apixaban in human plasma and its application to pharmacokinetics studies in the Indian population. Anal Methods 2021; 13: 3693–3704.

References

[1] Yeh CH, Hogg K, Weitz JI. Overview of the new oral anticoagulants opportunities and challenges. Arterioscler Thromb Vasc Biol 2015; 35:1–9.

[2] Sarich TC, Seltzer JH, Berkowitz SD, et al. Novel oral anticoagulants and reversal agents: Considerations for clinical development. Am Heart J 2015; 0:1–7.

[3] Olson H (2016) Advantages and disadvantages of novel oral anticoagulants. https://www.dicardiology.com/article/advantages-and-disadvantages-novel-oral-anticoagulants.

[4] Nouman EG, Al-Ghobashy MA, Lotfy HM. Development and validation of LC–MSMS assay for the determination of the prodrug dabigatran etexilate and its active metabolites in human plasma. *J Chromatogr B* 2015; 989: 37–45

[5] Saffian SM, Zhang M, Leong Chin PK, et al. Quantification of dabigatran and indirect quantification of dabigatran acylglucuronides in human plasma by LC–MS/MS. *Bioanalysis* 2015; 7(8): 957–966.

[6] Li J, Fang J, Zhong F, et al. Development and validation of a liquid chromatography/tandem mass spectrometry assay for the simultaneous determination of dabigatran etexilate, intermediate metabolite and dabigatran in 50μL rat plasma and its application to pharmacokinetic study. *J Chromatogr B* 2014; 973: 110-119.

[7] Antovic JP, Skeppholm M, Eintrei J, et al. Evaluation of coagulation assays versus LC-MS/MS for determinations of dabigatran concentrations in plasma. Eur J Clin Pharmacol 2013; 69(11): 1875–1881.

[8] Delavenne X, Moracchini J, Laporte S, et al. UPLC MS/MS assay for routine quantification of dabigatran – A direct thrombin inhibitor – In human plasma. *J Pharm Biomed Anal* 2012; 58: 152–156.

[9] Rohde G. Determination of rivaroxaban–a novel, oral, direct Factor Xa inhibitor–in human plasma by high-performance liquid chromatography-tandem mass spectrometry. J Chromatogr B 2008; 872:43–50.

[10] Iqbal M, Khalil NY, Imam F, et al. A validated high-throughput UHPLC-MS/MS assay for accurate determination of rivaroxaban in plasma sample. J Thromb Thrombolysis 2014; 39(1):79–88.

[11] Celebier M, Recber T, Kocak E, et al.. Determination of Rivaroxaban in Human Plasma by Solid-Phase Extraction–High Performance Liquid Chromatography. J Chromatogr Sci 2015; 54(2):216-2020.

[12] Delavenne X, Mismetti P, Basset T. Rapid determination of apixaban concentration in human plasma by liquid chromatography /tandem mass spectrometry: application to pharmacokinetic study. J Pharm Biomed Anal 2013; 78(79):150-153.

[13] Pursley J, Shen JX, Schuster A, et al. LC–MS/MS determination of apixaban (BMS-562247) and its major metabolite in human plasma: an application of polarity switching and monolithic HPLC column. Bioanalysis 2014; 6(15): 2071-2082.

[14] Ioan T, Daniela SP, Timea SX, et al. Determination of Apixaban Levels in Human Plasma by a High-Throughput Liquid Chromatographic Tandem Mass Spectrometry Assay. Romanian Review of Laboratory Medicine 2015; 1(23):115-126.

[15] Xu, Alan Q, Madden, Timothy L. LC-MS in Drug Bioanalysis. 1st ed. USA: Springer; 2012. 472 p.

[16] USFDA: Draft Guidance on Apixaban; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/psg/Apixaban_oral%20tablet_NDA%2020215 5_RV05-17.pdf

[17] USFDA: Draft Guidance on Rivaroxaban; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/psg/Rivaroxaban_oral%20tablet_22406_RC09 -15.pdf

[18] USFDA: Draft Guidance on Dabigatran Etexilate Mesylate; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/psg/Dabigatran%20etexilate%20mesylate_oral %20capsule_NDA%20022512_RV05-17.pdf

[19] EMA: Apixaban product-specific bioequivalence guidance; Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/apixaban-film-coated-tablet-25-5-mg-product-specific-bioequivalence-guidance_en.pdf

[20] EMA: Rivaroxaban product-specific bioequivalence guidance; Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/rivaroxaban-film-coated-tablets-25-10-15-20mg-product-specific-bioequivalence-guidance_en.pdf

[21] EMA: Dabigatran etexilate product-specific bioequivalence guidance; Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/dabigatran-etexilate-hard-capsule-75-mg-110-mg-150-mg-product-specific-bioequivalence-guidance_en.pdf